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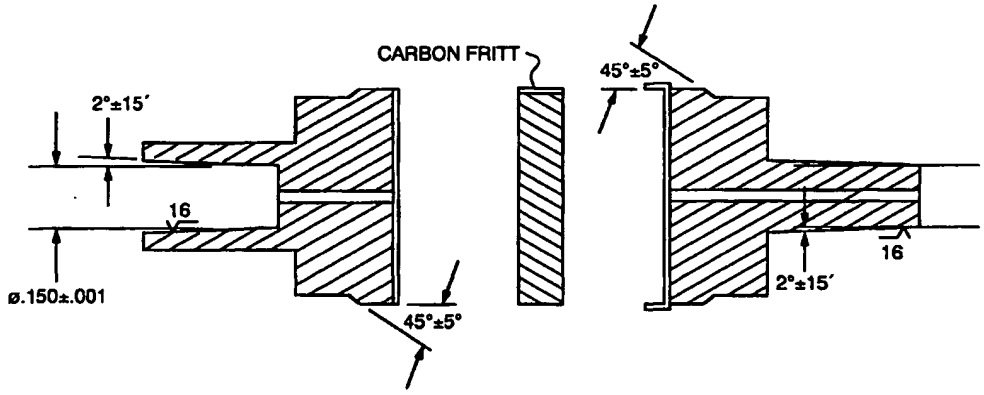

(57) Abstract

A sample pre–separation and concentration system for detecting low levels of analytes in highly complex mixtures includes a plurality of trapping columns having a high selectivity for classes of compounds of interest, upstream of a standard separation column. The system may be used, for example, to measure urinary 8 hydroxy 2'deoxyguanoisine.

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ELECTROCHEMICAL ANALYSIS SYSTEM

The present invention relates to improvements in analytical techniques. The invention has particular utility in connection with determination of low levels of analytes in highly complex mixtures, and will be described in connection with such utility, although other utilities are contemplated.

The determination of very low levels of specific analytes in complex mixtures of hundreds of possible interferences is a classical problem in analytical chemistry. For example, in recent developments in the assessment of oxidative stress and in the assessment of genetic damage from inherited and environmental factors, the measurement of urinary 8 hydroxy 2' deoxyguanosine (8OH2'dG) and other similar purine and pyrimidine indicators of DNA damage has developed as a potentially useful diagnostic therapy directing tool. These measurements have been used in clinical research in the areas of neurological disorders, cardiovascular problems, cancer, and assessment of biological environmental risk.

The overall utility of such measurements is compromised, however, by the lack of reliable measuring technology that will allow the intercomparison of values among different laboratories and studies. While several methods incorporating multiple separations and finally analytical techniques of liquid chromatography with electrochemical detection or gas chromatography, or mass spectroscopy have been published, they all suffer from problems of reliability, certainty of the analyte measured, complexity of preparation and manipulation, short and long term accuracy and precision, and cost. One of the most commonly cited techniques has been liquid chromatography with electrochemical detection employing a variety of preparation and concentration procedures and/or automated column switching. In automated column switching a portion of the eluent band from a first column is trapped in an injection loop and then transferred to a second column with different characteristics of separation. While some researchers reportedly have obtained reliable information with these techniques, the procedures are both fragile and prone to individual specific errors. This is because of the small non-quantitative and variable amounts trapped from the band and from the first column (typically

less than 10%), and because of the highly variable nature and high level of interfering species at the typical levels of ca 5ng/ml of analyte of interest.

It is thus an object of the invention to overcome the aforesaid and other disadvantages of the prior art. Another object of the present invention is to provide an analytical technique for determining low levels of analytes in complex mixtures. A more specific object of the invention is to provide an analytic technique for analyzing 8 hydroxy 2' deoxyguanosine in biological samples.

In order to effect the foregoing and other objects of the invention, in accordance with the present invention, there is provided a sample pre-separation and concentration system comprising a plurality of trapping columns having a high selectivity for classes of compounds of interest, upstream of a standard separation column. For example, as applied to the analysis for 8OH2d'G in urine and other biological samples, the basis of the invention is the replacement of the injection loop from the first column with one or more small porous carbon columns that have a very highly selectivity for purines, and certain other classes of compounds such as aromatic amines and nitro compounds and certain flavones flavenoids and other highly conjugated species. These columns have sufficient selectivity that they can trap essentially the entire eluting band containing the analyte of interest in a very small, e.g. 20-250ul volumes, and then be flushed for a relatively long time by a second eluting buffer to remove almost all species but the analyte of interest. The porous carbon column is then switched to the head of a second separation column employing a buffer identical to the flushing buffer except for a displacing agent that strips the analyte of interest from the carbon in a sharp peak that is compatible with optimum separation characteristics of the second column. By way of example, for 8OH2'dG, the displacing agent of preference is Adenosine, a compound of similar structure but not electroactive. The principle of similarity by non-detectability is general for the third buffer additive. For example, phenylalanine may be used to displace nitrotyrosine.

For a further understand of nature and objects of the present invention, presence should be had to the following detailed description taken in conjunction with the accompanying drawings wherein:

Fig. 1 is a schematic view of one form of sample separation and analysis system in accordance with the preferred embodiment of the invention;

Fig. 2 is a side elevational view, in cross section, showing details of a preferred form of sample preparation column useful in accordance with a preferred embodiment of the present invention;

Fig. 3 is a side elevational view, in cross section, of a separation and/or testing column controlled as an electrochemical cell useful in accordance with a preferred embodiment of the invention; and

Fig. 4 is a series of graphs showing the current over time of an electrochemical analysis in accordance with the present invention.

Further understanding of the features and advantages of the present invention will be had from the following detailed description of the invention, which illustrates the analysis of
8 hydroxy 2' deoxyguanosine (8OH2'dG) in urine. It will be understood, however, that the invention advantageously may be employed for analyzing other analytes in complex mixtures.

A key feature and advantage of a preferred embodiment of the invention involves the use of unique carbon packed cells for separating and concentrating analytes of interest. In order to insure essentially one hundred percent trapping of the analyte of interest in very small volumes, two technical problems have been addressed. One critical design and construction issue for carbon packed cells is the sealing of the edge of the carbon cylinder in such a way that no flow path can be established that does not go through the mass of the carbon and that the carbon is not crushed. For carbon packed cells of either separate particles or sintered carbon material which operate at relatively low pressures of 5-15 bar this can be achieved by diverse techniques. For example, (as in Figure 2) heat shrink tubing,

microwaving of pressfit parts and ultrasonic welding of pressfit parts are all acceptable techniques. However, for columns which have to operate up at higher pressures, e.g. 100-400 bar, the design and construction process is more complex. To accomplish this in accordance with one embodiment of the invention, a chemically inert plastic sleeve under compression operating essentially as a highly viscous fluid is employed. The plastic characteristics necessary are chemical inertness and a compression deformation point below the typical 689 bar deformation point of the porous carbons. A low creep rate at room temperature and a high coefficient of thermal expansion is also desirable. A range of high molecular weight linear polyethylenes are commercially available and advantageously may be employed.

High pressure resistant packed carbon cells, useful as pre-separation cells in accordance with the present invention, are prepared as follows: Referring to Fig. 3, cylindrical carbon frits (2) are inserted into a plastic sleeve (4) of 1.28 I.D. centimeter diameter. Plastic sleeve (4) is formed of a high molecular weight linear polyethylene preferably with a melt point of 128°C, and having compression deformation point of 621 bar and a coefficient of expansion of 12.95cm/cm/°C. The assembly then is cooled in dry ice acetone. After insertion into the dry ice acetone, end pieces (1) formed of another chemically inert plastic material, and having a lower coefficient of expansion than the coefficient of expansion of the plastic sleeve (4), for example, polyether ether ether ketone (PEEK) are assembled onto sleeve (4), and tightened to the point of touching the carbon, and the assembly is then removed from the dry ice acetone. (The cooled resulting assembly is then inserted in a metal sleeve 3 of 1.27 I.D. centimeter diameter.) The assembly is capped and allowed to warm to room temperature. When the piece comes to room temperature, the differential expansion of the polyethylene sleeve as compared to the PEEK fittings and the metal retaining sleeve seals the fittings to the sleeve and brings the internal pressure of the assembly to ca 552-621 bar and maintains the seal

at about 414 bar internal pressures that are the maximum expected in chromatographic separations.

Another critical design characteristic is the selection of the base porous carbon material and the treatment of the carbon to achieve a balance of desired characteristics. These characteristics include:

1. Low back pressure,
2. A high number of theoretical plates,
3. Control of the oxidizing and reducing potential of the surface,
4. High capacity for adsorption, and
5. High and specific selectivity for different classes of compounds.

Characteristics 1 and 2 are in opposition. A high number of plates implies typically a small pore size which implies a high back pressure. Optimization is achieved at any pore size by having highly uniform pores and as small an amount of supporting matrix (as large a number of pores as possible). In the current invention this is achieved as follows:– First, attention is given to selection of the base carbon material. We have found that 8μ to 12μ particle size carbon sintered into a cohesive material with 0.5-1μ pore size works best. In this regard, we have selected carbon available from Poco Union Carbide Inc. under the designation PS2. With suitable cleaning, this material can be used directly to advantage. However, in order to improve performance, we also pre-condition the carbon by selectively etching the material to make the pore size more uniform and increase the net porosity. Selective etching can be accomplished by either of two processes with essentially equivalent results. One process involves electro-chemically pulsing the carbon in an acid saline solution. Preferably, the carbon is subjected to pulsing at 0-1.4V. vs. SCE in a saturated saline solution of 1-2 M in HCl for 24-48 hours. The other process utilizes a modification of the water gas reaction in which the carbon is heated in an oxygen free steam atmosphere, e.g. at 370-480°C for 24-240 hours. In the first case the etching is controlled by monitoring the total coulombs passed during the process and by monitoring the increase in capacitive current. In the

second case by monitoring the volume of the H_2 and CO produced in the reaction. Both processes significantly increase the uniformity of the pores, and thus the capacity of the carbon materials, reduce the back pressure and increase somewhat the number of separation plates. Both processes can be used in series, and also advantageously may be employed to pre-condition bulk carbon and graphite that is not initially highly porous.

The control of the redox status of the carbon is critical both because of the possibility of loss of a fragile analyte by oxidation and because the redox potential has a significant effect on the selectivity and retentivity of particular analytes. This can be achieved initially by the control of the selective etching process. The water gas reaction creates inherently a reductive surface ca -.2v vs a SCE and the electrochemical process can be held at a reducing potential after completion to achieve the same effect. Control of the redox potential can periodically be maintained for 2-8 days by chemical poising with such agents as ascorbate, borohydride or dithiothriotol. Activation and control of redox potential for the single use packed carbon cells can be established initially for production purposes by bulk refluxing of the carbon in propanol HCl. For more precise control of the redox potential of the columns, the carbon is controlled as a test electrode as shown in Figure 3. Control of redox potential whether chemical or electrochemical controls the selectivity of certain compounds. Reducing potentials of -100-0 mv vs SCE give approximately twice the retention time for purines as potentials of 300-400 mv vs SCE.

Tests of the carbon packed cells utilizing 2D gel electrophoresis also indicate that the redox potential affects the retention of certain classes of proteins and large macromolecules.

The present invention, which provides a sample preparation and concentration system which may be fully automated, and if desired, integrated into a fully automated assay system, will now be described in connection with the

separation and measurement of 8OH2'dG in urine. Referring in particular to Figs. 1 and 4 of the drawings, the overall process is as follows:

Aqueous based buffers were provided as follows:

Buffer A: 4% Methanol, 0.1 M Lithium Acetate at pH 6.5, balance in water.

Buffer B: 4.5% Acetonitrile, 2% Acetic Acid, 0.1 M Lithium Acetate at pH 3.4, balance in water.

Buffer C: 90% Propanol, 5% Acetic Acid, 0.2 M Lithium Acetate, balance in water.

Buffer D: 4.5% Aceonitrile, 2% Acetic Acid, 0.2 M Lithium Acetate at pH 3.4, 1.5 G/l Adenosine and 500ul/l of Buffer C.

Selection and composition of buffers is directed by several considerations. Buffer A should be neutral to slightly basic in order to be compatible with a dilution buffer that will dissolve particulates in the sample. Critical to the selection of the diluent is the dissolution of any particulate material precipitated from a urine sample which by co-precipitation mechanisms will contain a variable fraction of the 8OH2'dG. A secondary consideration is matching the ionic strength and organic modifier concentration of the buffer A to achieve long term stability of retention times on the first column. Buffer B and D should be exactly matched except for the carbon eluting compound and small additions of buffer C to buffer D in order to avoid large void effects and small baseline disturbances. Buffers B and D should be of sufficiently high organic modifier composition to clean the carbon of possible interferences in a reasonable time, and buffers B and D should contain a different organic modifier and be at a different pH from buffer A to enhance separation of any possible interferences. Buffer C should be of sufficient cleaning power to remove any residual poisoning agents from the carbon over extended periods of

use. Finally, since low levels of organic modifier are involved, all buffers should be bacterio and fungistatic.

Figure 1 shows the valving and switching setup for transferring various segments of the sample. The timing of events is begun from the injection of the sample by the autosampler AS onto the head of the first column C18/1 is as follows:

Time 0. CONDITION 1: c18/1 at a flow rate of 0.9ml/min of buffer A delivered by pump P1; Carbon 1 and Carbon 2 at a flow rate of 1ml/min of buffer B delivered through a low pressure switching valve LPV and pump P2; column C18/2 at a flow rate of .9 ml/min of buffer D delivered through pump P3.

Time 11.3-12.8 min CONDITION 2: valve 2 switches directing buffer A through C18/1 and carbon 1 and carbon 2.

Time 12.8-17.0 valve 2 switches back to condition 1 flushing the trapped band of sample on carbon 1 and carbon two with buffer B

Time 13.2-29 min CONDITION 4 valve 1 switches to reverse the flow of buffer A through column C18/1.

Time 17.0-17.8 CONDITION 5 valve 3 switches to direct the flow of buffer D through carbon 2 onto column C18/2.

Time 17.8 valve 3 switches back to condition 4.

Time 18-29 l pv switches to deliver buffer C to carbon 1 and carbon 2 to clean the carbons and extend their longevity cycle between maintenance events.

Time 29-40 system in condition 1.

Time 40 initiate next cycle.

Cell 1 is a Model 5010 multiple electrode electrochemical detection cell, available from ESA, Inc. The cell comprises a multiple electrode coulometric cell, and is used for monitoring the output of column C18/1. It is used primarily for calibration of retention time and to measure major constituents in a sample either as a quality control measure or to obtain normalizing values for the primary analyte in the sample. Typical settings are T1 400 mv, T2 700 mv. Cell 2 is a series

combination of an ESA Model 5020 conditioning cell, and an ESA Model 5011 analytical cell. The conditioning cell is typically set at 10 mv and the 5011 cell at T1 20 mv T2 180 mv.

Figure 4 shows the typical output of an assay for a 1 ng/ml and 20 ng/ml 8OH2'dG calibration standard, a urine sample and the same sample augmented with 10 ng/ml of 8OH2'dG. Samples are prepared by dilution 1:1 with a two fold concentration of buffer A. Sixty-nine samples were run over two days including 48 samples, 5 calibration standards at 20 ng/ml, 4 duplicates 4 samples spiked at 10 ng/ml, 2 high and 2 low quality control pool urines, and a regression line set of 4 standards at 1, 3, 10 and 30 ng/ml. Values were calculated from bracketing 20 ng/mml standards at position 1, 18, 35, 52 and 69, duplicates and spikes are run 30 positions apart during the assay, and regression line standards at positions 17, 34, 51 and 68 are treated s samples. Over 6 runs, the typical precision of duplicate pairs at a level of 4 ng/ml is $\pm$0.04 or 1% rsd, recoveries of spiked samples is 100% $\pm$ 1.8% rsd, low QC pools are 2.61$\pm$ 0.04, high AQC pools are 8.73$\pm$ .008, the precision of standards treated as duplicate pairs is 20.00$\pm$ 0.19, and the regression line slope is 0.998$\pm$ .005.

The invention is susceptible to modification. For example, the pre-treated porous carbon matrix made as above described advantageously may be used in an off- line sample preparation cartridge. By way of example, a disk of 1.27 cm diameter 0.38 cm thick pre-conditioned, packed carbon frit 50 was trapped in a non-contaminating polypropylene and polyethylene housing 52 adapted with a Luer type fitting 54. The resulting cartridge may be used either alone to trap a compound of interest from a sample, or in conjunction with a C18 or other solid phase extraction device to obtain a secondary level of separation. For example, for assay or urinary 8OH2'dG the following sample preparation protocol has been used:

1 ml of urine diluted 1:1 with pH8 0.1M Li Acetate (aqueous solution) is passed through a C18 solid phase separation device;

2 ml of 1% Methanol in water is passed through the C18;

1 ml of 12% Methanol in water is passed through the C18 and the packed carbon cell in series to elute the 8OH2'dG from the C18 and trap it on the packed carbon; and

2-3 ml of 12% Methanol in water is then passed through the packed carbon cell alone.

The 8OH2'dG is then eluted from the packed carbon with 500 ul of 0.003 g/ml Adeosine in the running buffer for a following LCEC assay. Optionally, the first 200 ul of the eluent can be discarded and the next 300 ul used for the assay.

Results from the off line assay are equivalent to those of the automated on line assay; however, because of the decreased discrimination of the C18 solid phase extraction vs. that of the first stage C18 column, there are more peaks that occur in the final chromatogram and more care must be taken to avoid and detect possible interferences.

Various changes may be made without departing from the spirit and scope of the present invention.

CLAIMS

1. A method of pre-treating a porous carbon or graphite material for use in a subsequent chemical analysis of complex mixtures characterized by selectively etching the carbon or graphite under controlled conditions so as to increase the net porosity of the material, and render the pore size more uniform.

2. A method according to claim 1, characterized by the fact that said selective etching comprises subjecting the carbon or graphite material to timed pulses in a voltage or current controlled cell, wherein said timed pulses preferably comprise controlled anodic potential pulsing, if desired, conducted in an aqueous acidic solution, preferably, an acidic halide.

3. A method according to claim 1, characterized by the fact that said carbon or graphite materials are selectively etched using deoxygenated steam.

4. A high pressure resistant column comprising a porous core material 2, surrounded by a sleeve 3, 4, and fittings 1 on the ends of said sleeve, said fittings and said sleeve having mismatched thermal rates of expansion.

5. A column according to claim 4, characterized by one or more of the following features:

(a) wherein said porous material 2 comprises a carbon or graphite cylinder and the sleeve 3, 4 comprises a high molecular weight polyethylene;

(b) wherein said end fittings 1 are formed of a polyether ether ketone;

(c) wherein said fittings 1 are compression fitted to said sleeve 3, 4;

(d) wherein said fittings 1 are threaded onto said sleeve 3, 4;

(e) wherein said porous core material 2 comprises carbon or graphite; and

(f) wherein said sleeve 3, 4 comprises a hollow cylinder.

6. A method of assembling a high pressure resistant column as claimed in claim 4, characterized by loading said porous core material into said sleeve, cooling the loaded sleeve to below room temperature, mounting the fittings on the end of said sleeve, and permitting the resulting assembly to return to room temperature.

7. A method of assembling a high pressure resistant column as claimed in claim 4, characterized by providing a sleeve filled with porous core material, heating said end fittings and mounting the heated end fittings to the sleeve, and permitting the resulting assembly to cool to room temperature.

8. A method for analyzing an analyte of interest in a sample solution comprising a complex mixture of materials, characterized by passing the sample solution through one or more trapping columns wherein a band of the material of interest can be trapped, washing the trapped contents and passing the resulting wash solution to a separation column where the analyte of interest may be further concentrated, washing the eluent from second trapped column, and passing the resulting solution to an analytical column where the analyte of interest may be detected and measured.

9. A method according to claim 8, characterized by one or more of the following features:

(a) wherein said sample material comprises 8OH2'dG in urine;

(b) wherein said trapping column is comprised of carbon particles;

(c) wherein said trapping column is comprised of porous carbon;

(d) wherein said sample comprises DNA adducts;

(e) wherein said sample comprises hydroxylated products of cytosine, uracil, guanine, guanosine and adenosine, in urine; and

(f) wherein said sample comprises nitro substituted products in urine.

10. An analytical reagent comprising Lithium ion as bacteriostatic/ fungistatic agent; or methanol and lithium acetate in water; or acetonitrile, acetic acid and lithium acetate in water; or propanol, acetic acid, and lithium acetate in water; or aceonitrile, acetic acid, lithium acetate, adenosine, and propanol in water.

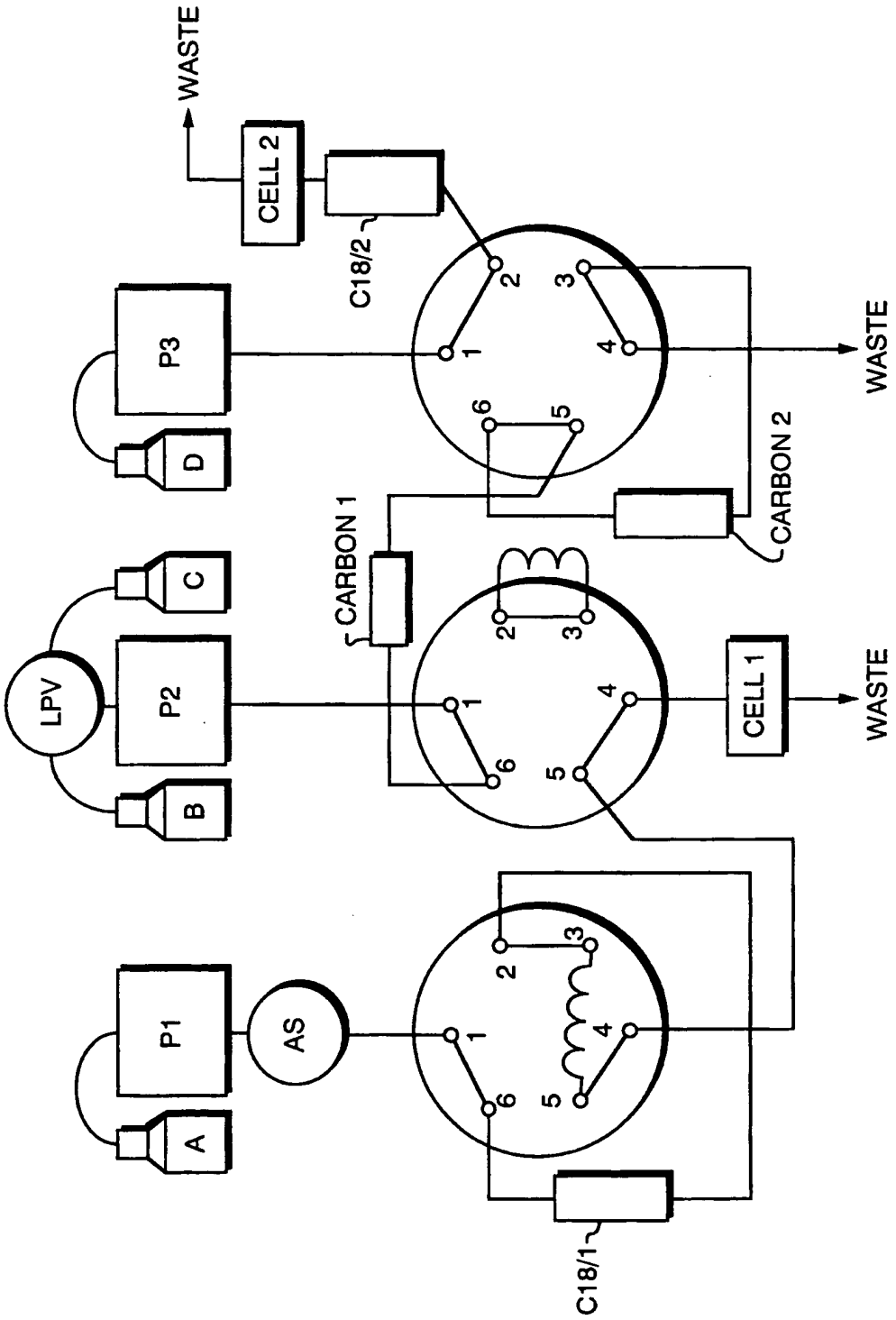
WASTE
CELL 2
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FIG. 1

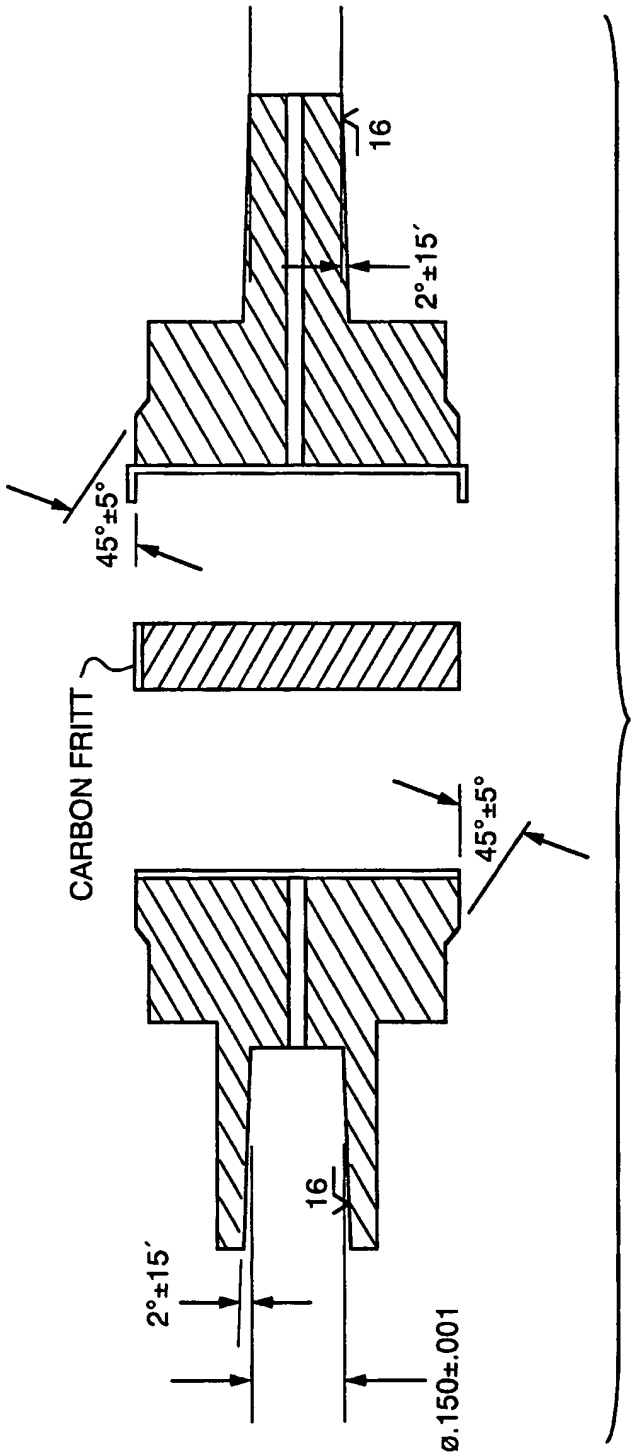

FIG. 2

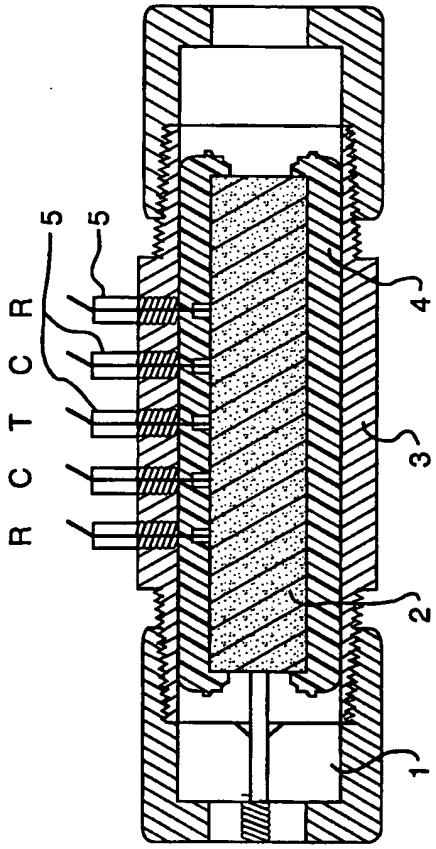

FIG. 3

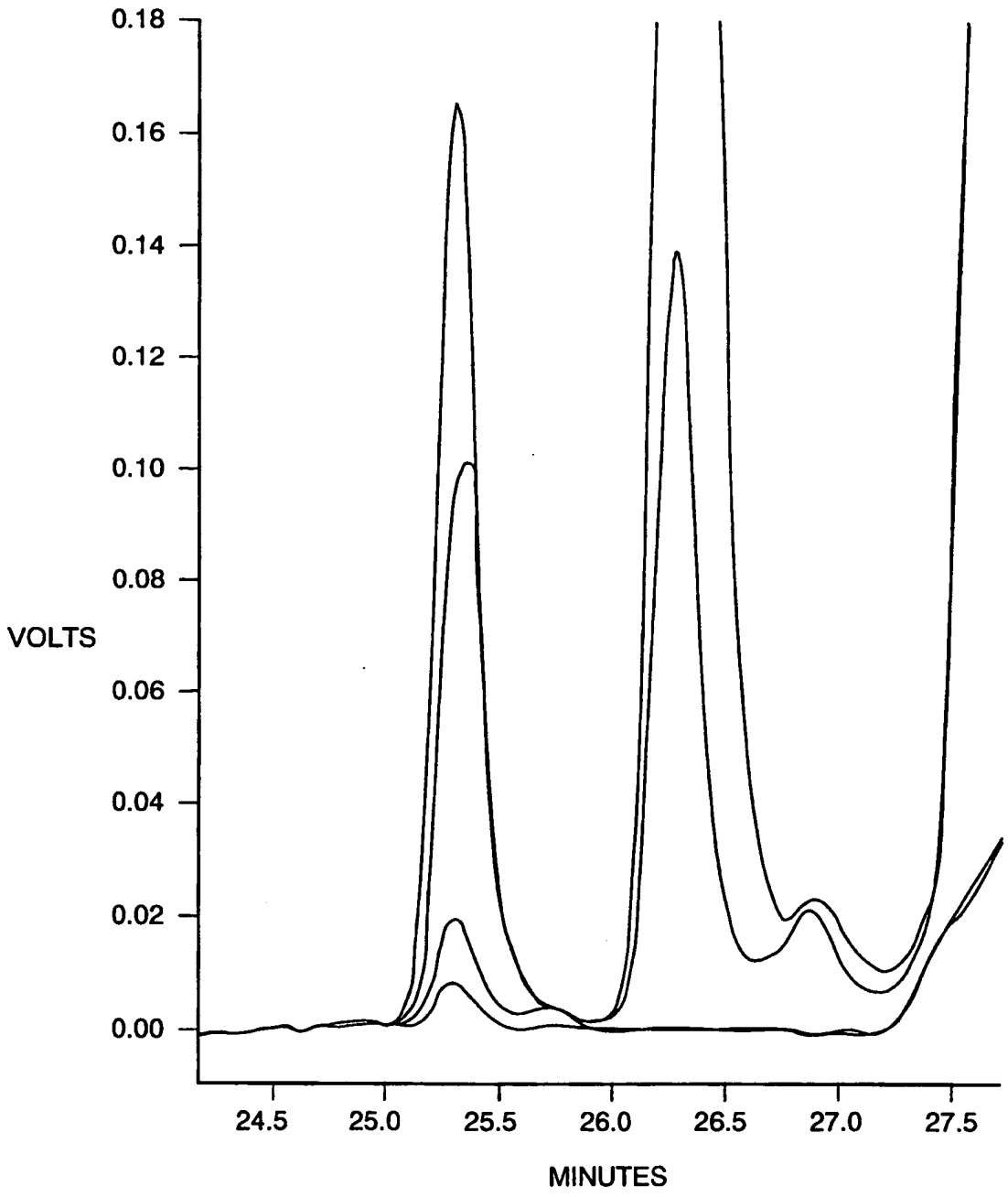
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
VOLTS
24.5
25.0
25.5
26.0
26.5
27.0
27.5
MINUTES

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22275

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N30/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP 0 365 327 A (UNILEVER PLC ;UNILEVER NV (NL)) 25 April 1990 see page 2, line 24-48 ---	1
A	US 4 263 268 A (KNOX JOHN H ET AL) 21 April 1981 see page 1, line 5-16 see column 1, line 64 - column 2, line 59 ---	1
A	US 5 358 802 A (MAYER STEVEN T ET AL) 25 October 1994 see column 7, line 1-9 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1999	23/04/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Im ational Application No
PCT/US 98/22275

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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A	see page 401, column C1, paragraph	1,9
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2

INTERNATIONAL SEARCH REPORT

Information on patent family members

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